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A NEW METHOD FOR CYTOLOGICAL DIAGNOSIS OF PULMONARY CANCER

By

LUDWIG VON BERTALANFFY AND FELIX D. BERTALANFFY

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A NEW METHOD FOR CYTOLOGICAL DIAGNOSIS OF PULMONARY CANCER

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A NEW METHOD FOR CYTOLOGICAL DIAGNOSIS OF PULMONARY CANCER*

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The acridine-orange fluorescence method, first developed for exfolia- ve cytology in gynecology,[1-6] has been applied to the diagnosis of alignancies of the respiratory system.

The fluorescence method uses only one dye in a simple procedure, sulting in a highly polychrome picture demonstrating all essential diag- stic criteria of malignancy.

In addition to the conventional criteria of the nucleus and the cell as a ole, the method introduces a new diagnostic category, that is, changes the RNA content of the cytoplasm that are not recognized in other utine clinical methods, but appear to be a primary factor in malignant owth.

Proliferating malignant cells are characterized by an RNA content that far exceeds that of the nonmalignant cells of origin.[7-9] These cyto- asmic changes are readily observed by the fluorescence method.

The method is based upon the metachromatic properties of the fluores- nt dye, acridine orange, leading to differential staining of the two types nucleic acids of the cell.[10] Acridine orange demonstrates DNA of the cleus in green to yellow fluorescence, and RNA of the cytoplasm and cleolus, with increasing concentrations, in reddish brown to orange to d fluorescence. Cells lacking appreciable amounts of RNA (for example, perficial squamous cells) show green cytoplasmic fluorescence; cells th moderate amounts of RNA (for example, basal cells, macrophages) pear in reddish brown fluorescence. In contrast, suspicious and malig- nt cells that, owing to their high protein synthesis and mitotic activity, ve a high RNA content, manifest a striking orange to flaming red orescence.

In comparative investigations[3,4,6] the acridine-orange fluorescence thod was found to be at least as reliable as any of the present-day thods of exfoliative cytology. It requires only six minutes for staining d, on the average, three minutes for screening the specimen. Because of the striking differences between normal and malignant cells shown b this technique, malignant cell elements can be recognized even by ob servers with little cytological training. It is therefore evident that th method offers great advantages for screening programs, routine work i hospitals, and in private practice.

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*This paper is based on presentations given at the Second Workshop Conference on ng Cancer of the American Cancer Society (L. v. B.) and to the American Association Anatomists (F. D. B.).

†The work of this author is supported in part by research grants from the National ncer Institute of Canada, Toronto, Ont., Canada, and The Damon Runyon Fund for Cancer search, New York, N. Y.

Material and Method

Routine material comprising sputums, bronchial secretions obtained b bronchoscopy, and pleural fluids was used in the present investigation The diagnoses were classified as *negative* (no cytological changes o slight metaplasia due to such factors as inflammatory processes), *suspicious* (atypical cells with increased cytoplasmic fluorescence), an *positive* (cells with pronounced orange to flaming red cytoplasmic fluorescence and obvious nuclear changes). The diagnoses obtained with th fluorescence method were counterchecked in every case on correspondin hematoxylin-eosin stained specimens.

In the present study, a Reichert Fluorex unit was used in a darkene room. Photomicrographs were taken routinely with a Leica attachment.

Technique. Specimens were processed as follows:

(1) Smears were fixed in ether-alcohol 1:1 for 5 to 15 min. They ma remain indefinitely in the fixative without impairment of their fluorescen qualities. If fresh specimens are processed immediately, fixation may b omitted.

(2) The samples were hydrated by dipping into 80, 70, and 50 per cen alcohol and distilled water.

(3) The samples were rinsed briefly in acetic acid (1 per cent) an twice in distilled water.

(4) The samples were stained for 3 min. in 0.01 per cent acridin orange.† A stock solution of acridine orange (0.1 per cent in distille water) is diluted according to need with phosphate buffer *p*H 6 (1/1 M KH_2PO_4 + 1/15 M Na_2HPO_4 mixed in proportion to obtain *p*H 6 an controlled with a *p*H meter).

(5) The smears were washed in the phosphate buffer indicated for a least 1 min. to remove excess dye. They may remain in the buffer solution for several hours if smears are stained in batches.

(6) Differentiation in $CaCl_2$ (1/10 M) from 1 to 2 min. until the nucle appear in a clear translucent green.

(7) The smears were rinsed in the phosphate buffer and covered with cover glass.

The solutions used were kept in Coplin jars, the buffer for the las nse in a polyethylene squeeze bottle for quick application.

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*Other types of fluorescence equipment (Zeiss-Winkel, Ortholux, and others) a equally suitable.

†Gurr, Esbe Laboratory Supplies, Toronto, Ont., Canada.

Slides may be returned into the ether-alcohol fixative and restained ccording to the above acridine-orange procedure or, after destaining, ley may be restained by any other histological technique.

Results

In the following, the cell types observed in sputums, bronchial secreons, and pleural fluids are described as they appear with the acridineange fluorescence technique.* The terminology used here is based upon evious histological and histochemical investigations.[12, 13]

Normal Cytology

Sputums and bronchial secretions. Squamous epithelial cells derived om the stratified squamous epithelium of the esophagus, pharynx, and al cavity occurred in two different morphologic forms. If they derived om the superficial layers of the epithelium, the cells were large and lygonal, with faint green, transparent cytoplasm and yellow pyknotic uclei (FIGURE 1). The cells from the basal layers were smaller, with ellowish brown cytoplasm and large greenish yellow nuclei, often with nspicuous green chromatin granules. If present, the sex chromatin was early visible adjacent to the nuclear membrane.

Ciliated columnar cells of the trachea, bronchi, and larger bronchioles ppeared singly or in groups, often embedded in mucous material. They ere slender cells with greenish nuclei, located at the cell base or in the iddle portion of the cells. The cytoplasm appeared reddish brown, and e brownish cilia were clearly visible (FIGURE 2).

Mucous goblet cells, also originating from the respiratory epithelium, owed reddish brown, often reticulated cytoplasm (presence of mucous aterial). The yellow, often triangular nucleus was situated in the basal rtion of the cell.

Basal cells of the pseudostratified epithelium occurred in multiple rms, flattened, triangular or oval, either singly or in small aggregates. the fluorescence picture, their cytoplasm was faintly reddish brown d their central nuclei appeared greenish yellow.

Owing to their similar appearance, cuboidal bronchiolar cells, derived m the lining of the smaller bronchioles, were in most cases difficult to stinguish from the basal cells of the pseudostratified epithelium with ich they are presumably contiguous.

Pulmonary macrophages, also called alveolar cells, dust cells, or

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[*] Some color microphotographs of cell types of exfoliated cells from various origins demonstrated by the acridine-orange fluorescence method, are reproduced in Bertalanffy d Bertalanffy.[11]

histiocytes, originate by mitosis in the alveolar walls, subsequentl desquamate, wander through the alveoli, where they phagocytose inhale material, including particulate matter and bacteria, and are eventuall extruded with the sputum. They are actively renewed by mitosis[12,13] (i the rat, at a rate of about 15 per cent per day), and large numbers of mac rophages are extruded from the respiratory tissue (in the rat, about 2 million per day). A minor pathway of extrusion of these cells is thei entrance into the perivascular and peribronchial connective tissue, wher they sometimes form pneumoconiotic foci. The presence of pulmonar macrophages in bronchial secretions and sputum is thus a normal occur rence and, indeed, is an indication of a satisfactory sample. In smea preparations, the macrophages are highly polymorphic in size and shape often contain vacuoles and phagocytosed material in the cytoplasm, an frequently are bi- or multinucleated.

With the fluorescence technique, these cells always show a reddis brown cytoplasm, and the nuclei varied from dark green to light green o yellow (if pyknotic; see FIGURE 3). In subchronic or chronic infection pulmonary macrophages, together with leukocytes, appeared in greatl increased numbers.

Pleural fluids. Mesothelial cells were rather fragile cells, irregular i shape, often with torn or fuzzy cytoplasm. They had faintly brownis cytoplasmic fluorescence and centrally located, oval, or crescent-shape green to yellow nuclei (FIGURE 7).

Pleural macrophages, presumably derived from the connective tissue o the visceral and parietal pleura, were usually round cells, sometimes wit reticulated cytoplasm. Normally, the cytoplasm displayed reddish brow fluorescence (FIGURE 7). If macrophages occurred in greatly increase numbers, as in chronic irritations, the cytoplasm appeared red, corresponding to the high mitotic rate in such instances. However, due to their normal morphology, these cells could be distinguished readily from suspicious cell elements. The nuclear fluorescence ranged from green to yellow. Macrophages sometimes occurred in the form of so-called signet-ring cells, with a large unstained lipid droplet pushing the nucleus toward the periphery of the cell. Sometimes macrophages were bi- or multinucleated and, occasionally, they formed syncytia (giant cells). A characteristic distinction between macrophages and malignant cells was that pleural macrophages occurred singly or joined into flat cell sheets; malignant cells usually appeared in irregular clumps.

"Active" respiratory epithelial cells, macrophages, and mesothelial cells often appeared in sputum, bronchial secretions, and pleural fluids during catarrhal and chronic irritations. These "active" cells were characterized by a larger RNA content and thus increased reddish cytoplasmic fluorescence; otherwise they showed normal morphology. Presumably wing to the irritation, the cells were stimulated temporarily to proliferate t a higher rate and, in such conditions, they often appeared in larger umbers. Shortly after the irritation had subsided or was cleared up, the ells then present again showed their normal brown or reddish-brown uorescence.

Leukocytes. Neutrophil granulocytes (polymorphs) occurred almost egularly in bronchial and tracheal secretions, because the respiratory assages are one pathway of their elimination. They leave the pulmonary apillaries by diapedesis, enter the alveolar walls and alveolar spaces, nd are eventually extruded with the sputum. They also penetrate the ronchial and tracheal epithelia and enter the lumen of the air-conducting ystem. Their number is increased greatly in catarrhal and acute inflam-ations. With the fluorescence microscope, the polymorphic (lobulated) uclei of these cells appeared bright green, whereas the cytoplasm emained unstained. In the cytoplasm of these actively phagocytic cells, arge numbers of red fluorescent bacteria were often visible.

Lymphatic elements (lymphocytes and monocytes) are also regularly resent in smear samples from the respiratory tract and pleural fluids. imilar to the neutrophils, these cells may enter the alveolar space of e lung or penetrate the epithelial lining of bronchi and trachea. They howed round to oval, green nuclei, often surrounded by a narrow rim of eddish-orange cytoplasm. Plasma cells were characterized by bright range fluorescent cytoplasm and round, eccentric nuclei appearing in reen fluorescence.

Malignant Cytology

Malignant cells from the respiratory system were generally character-zed by their intense cytoplasmic fluorescence, which was bright orange red in most types of bronchogenic carcinoma. Thus, malignant cells an be recognized readily even with low magnification (FIGURES 4 to 6). ome cell types frequently encountered are now discussed:

Epidermoid bronchogenic carcinoma presented large, flattened, squa-ous cells with orange cytoplasm and round or oval polymorphic nuclei, ften pyknotic and containing multiple nucleoli.

Cases considered to be adenocarcinoma of the lung showed the cyto-lasm in carmine red, possibly due to the presence of mucoproteins.

Anaplastic cells (oat cells) were small cells with bright orange to red toplasm and small yellow nuclei.

Malignant cells from metastatic tumors, present in pleural fluids, ppeared in most cases in the form of cell clumps. The cells varied reatly in size, were often vacuolated, and their nuclei were oval, rescent-shaped, or irregular. Tumor cells in pleural fluid were easily ecognized by their bright red cytoplasmic fluorescence (FIGURE 8).

SOME CELL TYPES OF THE RESPIRATORY SYSTEM AS SHOWN BY THE ACRIDINE-ORANGE FLUORESCENCE METHOD

FIGURE 1. Sputum, normal. Two large superficial squamous cells, with faint green cytoplasm, and several macrophages. Male, 32. ×250.

FIGURE 2. Bronchial secretion, normal. Numerous columnar cells from the respiratory epithelium. Cytoplasm reddish brown, nuclei yellow, cilia brown. Male, 52. ×250.

FIGURE 3. Bronchial secretion, normal. Macrophages, showing reddish-brown cytoplasm and yellow nuclei. Nuclei of leukocytes scattered over the picture. Male, 32. ×200.

FIGURE 4. Sputum with a group of large malignant cells. The bright red cytoplasm almost obscures the nuclei. Two normal squamous cells can be distinguished on the upper end of the malignant cell group. Male, 70. ×300.

FIGURE 5. Bronchial secretion, group of very suspicious cells. The cells, presumably of respiratory epithelial origin, show bright red cytoplasm and atypical morphology. Male, 52. ×250.

FIGURE 6. Bronchial secretion with four atypical cells. Rather intense reddish-brown fluorescence of the cytoplasm, large nuclei with pronounced nucleoli. Male, 60. ×600.

FIGURE 7. Pleural fluid, normal. Group of macrophages with faintly brown cytoplasm; some mesothelial cells, small lymphocytes (green nuclei with narrow rim of reddish cytoplasm), several neutrophils (green lobulated nuclei, unstained cytoplasm) in right upper corner. Female, 61. ×200.

FIGURE 8. Pleural fluid with a large group of malignant cells of pulmonary origin with intensely red cytoplasm. ×200.

(Microphotographs: Felix D. Bertalanffy)

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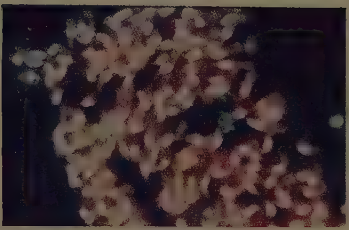

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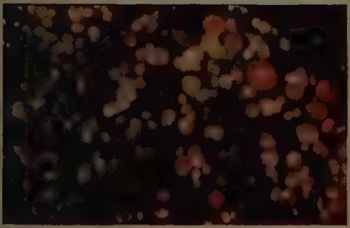

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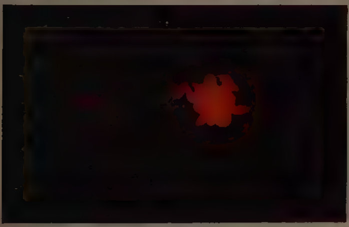

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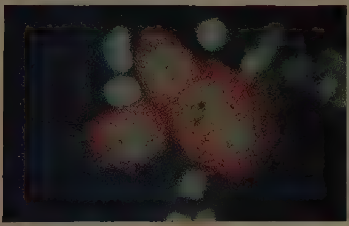

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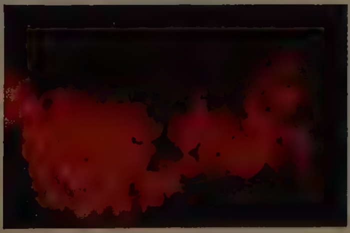

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In cases of degenerating tumors with loss of cytoplasm, the pleomorphic, bright green fluorescent nuclei made recognition of such cells possible.

A summary of the cell types present in exfoliative specimens of the respiratory system is given in TABLE 1.

Table 1

Exfoliative Cytology of the Respiratory System as Observed with the Acridine-Orange Fluorescence Method

1. Normal Cytology

Sputums, Tracheal and Bronchial Secretions

Cell type	*Origin and where found*	*Morphology*	*Appearance in acridine-orange fluorescence*
Stratified squamous epithelial cells from esophagus, pharynx, and oral mucosa	Normally in sputum only	From superficial layers: large, polygonal	Cytoplasm: faintly green Nuclei: green; yellow if pyknotic
		From basal layers: smaller, oval	Cytoplasm: yellowish brown Nuclei: green-yellow with chromatin particles
Ciliated columnar cells	Epithelium of trachea, bronchi, larger bronchioles	Single or in groups; slender, nucleus at cell base or in middle portion of cell	Cytoplasm: reddish brown Nuclei: green Cilia: brown
Goblet cells	Same	Seen less frequently (fragile); stouter shape; nucleus (often triangular) in basal portion of cell	Cytoplasm: reddish brown, reticulated (mucous material) Nuclei: yellow
Basal cells	Pseudostratified columnar epithelium of respiratory system	Either single or in small aggregates; various flattened forms; nuclei central and of uniform size	Cytoplasm: faintly reddish brown Nuclei: greenish yellow

TABLE I (Continued)

Cell type	*Origin and where found*	*Morphology*	*Appearance in acridine-orange fluorescence*
ulmonary macro-hages (alveolar ells, dust cells, istiocytes)	Formed by cell division in the alveolar walls; phagocytic; number increased in chronic infections	Polymorphic in size and shape; bi- and multinucleation; sometimes contain phagocytosed material in cytoplasm	Cytoplasm: reddish brown Nuclei: from dark green to yellow (if pyknotic)
	Pleural Fluids		
esothelial cells	Visceral and parietal pleura	Irregular, fragile cells, central oval nuclei	Cytoplasm: faintly brown Nuclei: green to yellow
leural acrophages	Connective tissue of pleura	Round cells, polymorphic, bi- and multinucleated; phagocytic; sometimes signet-ring cells	Cytoplasm: reddish brown; highly proliferating cells red Nuclei: green to yellow
	Leukocytes		
eutrophil granulo-ytes (polymorphs)	In bronchial and tracheal secretions, sputums, and pleural fluids. Number increased in inflammations	Polymorph lobulated nuclei	Cytoplasm: unstained Nuclei: bright green
ymphatic ele-ents (lympho-ytes, monocytes)	Same	Round to oval nuclei with varying amount of cytoplasm	Cytoplasm: sometimes a narrow orange rim Nuclei: green
lasma cells	Occasionally in bronchial secretions and sputums	Round eccentric nuclei; abundant cytoplasm	Cytoplasm: bright orange Nuclei: green

TABLE I (Continued)

Cell type	*Origin and where found*	*Morphology*	*Appearance in acridine-orange fluorescence*
		2. Malignant Cytology	
Epidermoid bronchogenic carcinoma	Sputum, bronchial secretions	Large, flattened squamous cells, nuclei round to oval	Cytoplasm: bright orange red Nuclei: yellow
Adenocarcinoma	Same	Pleomorphic cells, vacuoles	Cytoplasm: carmine-red (presence of mucoproteins) Nuclei: yellow
Anaplastic cells	Same	Small cells, nuclei of different size	Cytoplasm: bright orange Nuclei: yellow (pleomorphic)
Metastatic tumor cells	Pleural fluids	Great variation in size and shape of cells and nuclei	Cytoplasm: bright red Nuclei: yellow
Degenerating malignant cells	May appear in all malignancies	Pleomorphic, reduction of cytoplasm, vacuolation, naked nuclei	Naked nuclei: brilliant green, pleomorphic, larger than leukocytes

Summary

The rapidity, simplicity, and expediency of the acridine-orange fluorescence method, the striking appearance of malignant cells owing to their brilliant fluorescence, and the consequent facilitation of cytological diagnosis present definite advantages for lung cancer screening.

The fluorescence method permits easier recognition of cell types that may present difficulties in diagnosis of malignancies of the respiratory system. This applies particularly to Papanicolaou's "reserve cells"[14] or "basal undifferentiated cells" of the bronchial mucosa, which are the basal cells discussed above. In the fluorescence picture, normal basal

ells cannot present diagnostic difficulties because their cytoplasm ppears in reddish-brown fluorescence, as contrasted to the bright orange o red cytoplasm of malignant cells.

Similarly, in conventional procedure "atypical histiocytes probably onstitute the most frequent source of errors in the cytological interpreation of sputum specimens."[14] With the acridine-orange method, these ells also, appearing in reddish-brown fluorescence, are easily distinuished from the bright orange malignant cells.

In a study presently in progress, where routine samples from the respirtory system are investigated by the fluorescence method and independntly counterchecked with hematoxylin and eosin, the fluorescence ethod proved to be at least as reliable as the latter procedure. The dvantages of the fluorescence method are the reduced time for processg and screening, and probably a more clear-cut diagnosis.

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